Calcium Metabolism and Amylase Release in Rat Parotid Acinar Cells

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1. A method is described for the isolation of rat parotid acinar cells by controlled digestion of the gland with trypsin followed by collagenase. As judged by Trypan Blue exclusion, electron microscopy, water, electrolyte and ATP concentrations and release of amylase and lactate dehydrogenase, the cells are morphologically and functionally intact. 2. A method was developed for perfusion of acinar cells by embedding them in Sephadex G-10. Release of amylase was stimulated by adrenaline (0.1–10 μ M), isoproterenol (1 or 10 μ M), phenylephrine (1 μ M), carbamoylcholine (0.1 or 1 μ M), dibutyryl cyclic AMP (2 mM), 3-isobutyl-1-methylxanthine (1 mM) and ionophore A23187. The effects of phenylephrine, carbamoylcholine and ionophore A23187 required extracellular Ca^{2+} , whereas the effects of adrenaline and isoproterenol did not. 3. The incorporation of ^{45}Ca into parotid cells showed a rapidly equilibrating pool (1–2 min) corresponding to 15% of total Ca^{2+} and a slowly equilibrating pool (>3 h) of probably a similar dimension. Cholinergic and α -adrenergic effectors and ionophore A23187 and 2,4-dinitrophenol increased the rate of incorporation of ^{45}Ca into a slowly equilibrating pool, whereas β -adrenergic effectors and dibutyryl cyclic AMP were inactive. 4. The efflux of ^{45}Ca from cells into Ca^{2+} -free medium was inhibited by phenylephrine and carbamoylcholine and accelerated by isoproterenol, adrenaline (β -adrenergic effect), dibutyryl cyclic AMP and ionophore A23187. 5. A method was developed for the measurement of exchangeable ^{45}Ca in mitochondria in parotid pieces. Incorporation of ^{45}Ca into mitochondria was decreased by isoproterenol, dibutyryl cyclic AMP or 2,4-dinitrophenol, increased by adrenaline, and not changed significantly by phenylephrine or carbamoylcholine. Release of ^{45}Ca from mitochondria in parotid pieces incubated in a Ca^{2+} -free medium was increased by isoproterenol, adrenaline, dibutyryl cyclic AMP or 2,4-dinitrophenol and unaffected by phenylephrine or carbamoylcholine. 6. These findings are compatible with a role for Ca^{2+} as a mediator of amylase-secretory responses in rat parotid acinar cells, but no definite conclusions about its role can be drawn in the absence of knowledge of the molecular mechanisms involved, their location, and free Ca^{2+} concentration in appropriate cell compartment(s).

Secretion of saliva by rat parotid gland involves release of amylase, water and K^+ and can be modulated through three types of hormone receptors, the α - and β -adrenergic receptors and the muscarinic cholinergic receptor (for a review, see Schramm & Selinger, 1975). Release of amylase by rat parotid *in vitro* is strongly stimulated by β -adrenergic agonists (e.g. isoproterenol) and by dibutyryl cyclic AMP, but only weakly stimulated by α -adrenergic or cholinergic agonists (e.g. phenylephrine and carbamoylcholine) (Bdolah *et al.*, 1964; Babad *et al.*, 1967; Schramm, 1968; Amsterdam *et al.*, 1969; Selinger *et al.*, 1974; Butcher *et al.*, 1975; Mangos *et al.*, 1975b). Release of K^+ on the other hand is strongly stimulated by α -adrenergic and cholinergic agonists (Batzri *et al.*,

1973; Selinger *et al.*, 1973, 1974). Stimulation by adrenaline (a mixed agonist) of amylase release is blocked by propranolol (a predominantly β -antagonist), whereas stimulation of K^+ release is blocked by phentolamine (a predominantly α -antagonist). Stimulation of amylase release by β -adrenergic effectors or dibutyryl cyclic AMP does not require extracellular Ca^{2+} , and prolonged exposure of parotid tissue to chelating agents is required to inhibit amylase release with β -adrenergic agonists (Rasmussen & Tenenhouse, 1968; Selinger & Naim, 1970; Batzri & Selinger, 1973; Harfield & Tenenhouse, 1973; Dormer & Ashcroft, 1974). Stimulation of K^+ release by α -adrenergic or cholinergic agonists requires extracellular Ca^{2+} (Selinger *et al.*, 1973, 1974). β -Adrenergic agonists can increase the concentration of cyclic AMP in rat parotid, whereas α -adrenergic

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agonists are without effect (Malamud, 1972; Butcher *et al.*, 1975).

These findings have led to the suggestion that cyclic AMP may be responsible for stimulus-secretion coupling with respect to amylase release, and that Ca^{2+} may be responsible with respect to K^+ release (see, e.g., Schramm & Selinger, 1975). However, the cyclic AMP concentration in the parotid does not correlate well with the rate of amylase release (Butcher *et al.*, 1975), and the bivalent metal ionophore A23187 induces some release of amylase in addition to K^+ , provided that extracellular Ca^{2+} is present (Butcher, 1975). Ca^{2+} is accumulated by parotid mitochondrial and microsomal fractions (Selinger *et al.*, 1970; Dormer & Ashcroft, 1974) and ^{45}Ca is incorporated into these fractions in the tissue *in vitro*. Thus, if β -adrenergic agonists affect discharge of Ca^{2+} from either intracellular pool, participation of Ca^{2+} in amylase release would not be excluded by non-dependence on extracellular Ca^{2+} . The development of techniques for measurement of exchangeable Ca^{2+} of mitochondria in tissues has shown that adrenaline can decrease the exchangeable Ca^{2+} of mitochondria in adipose tissue and parotid (Severson *et al.*, 1976; Dormer & Ashcroft, 1974; Randle *et al.*, 1974). It may also decrease exchangeable Ca^{2+} in microsomal fractions of the parotid (Dormer & Ashcroft, 1974). Adrenaline is, however, a mixed α - and β -adrenergic agonist, and it is not known whether effects on mitochondrial and microsomal exchangeable Ca^{2+} are α - or β -adrenergic effects. Moreover, experiments with isolated parotid slices or pieces are complicated by the presence of sympathetic and parasympathetic nerve endings capable of releasing noradrenaline and acetylcholine.

In the present study methods have been developed for the isolation and perfusion of parotid acinar cells and for measurement of rates of release of amylase, ^{45}Ca uptake and ^{45}Ca efflux; parallel studies with parotid pieces for measurement of mitochondrial exchangeable Ca^{2+} are also reported. The effects of α - and β -adrenergic and cholinergic agonists, dibutyl cyclic AMP, ionophore A23187 and 2-isobutyl-1-methylxanthine have been studied in these systems. The terms ^{45}Ca uptake or incorporation are used to describe measurement that may reflect exchange or net uptake or both.

Experimental

Materials

Collagenase (type II), trypsin (type III), soya-bean trypsin inhibitor (type II-S) and bovine plasma albumin (fraction V) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Other biochemical reagents and enzymes were from Boehringer Corp. (London) Ltd., London W.5,

U.K., or from Sigma, with the following exceptions. Sephadex G-10, from Pharmacia (G.B.) Ltd., London W5 5SS, U.K., was acetylated with acetic anhydride as described by Reed (1973), and stored in aq. 50% (v/v) ethanol. Chelex-100 resin was from Bio-Rad Laboratories, Bromley, Kent, U.K. Ionophore A23187 was a gift from Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A. Ruthenium Red, adrenaline, Trypan Blue and carbamoylcholine were from British Drug Houses, Poole, Dorset, U.K. Isoproterenol (isoprenaline) was from Kodak Ltd., Kirkby, Liverpool, U.K. Phentolamine mersylate was from Ciba Laboratories, Horsham, West Sussex RH12 4AB, U.K. 3-Isobutyl-1-methylxanthine was a gift from Dr. W. Montague, Department of Biochemistry, University of Leicester, Leicester, U.K. Acetyl-CoA was synthesized by the method of Simon & Shemin (1953). MEM (minimum essential medium) amino acid supplement was from Flow Laboratories, Irvine, Ayrshire KA12 8NB, Scotland, U.K. Spectrographically standardized reagents were from Johnson Matthey Chemicals, London E.C.4, U.K. (MgSO_4 and CaCl_2) or British Drug Houses (KCl and LaCl_3). Other chemicals were of the purest grade obtainable.

Radiochemicals (^{45}Ca , $^3\text{H}_2\text{O}$, [^{14}C]sucrose and [$6,6\text{'-}^3\text{H}$]sucrose) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Preparation of parotid cells. Parotid glands were dissected from male albino Wistar rats of approx. 300g, which had had free access to water and diet 41B (Oxoid, London S.E.1, U.K.), and had been killed by stunning and decapitation. Initial attempts to isolate parotid cells were based on controlled digestion with collagenase and hyaluronidase by methods used for the preparation of pancreatic acinar cells by Amsterdam & Jamieson (1973). It was found difficult to control digestion of parotid with this technique, and a successful method was developed based on incubation with trypsin followed by collagenase and this method is described here. There was no advantage observed to the inclusion of hyaluronidase in the procedure.

The parotid glands of four rats were placed in basic preparation medium and cut into small pieces with scissors. The basic preparation medium consisted of bicarbonate-buffered saline (Krebs & Henseleit, 1932), but with only 1mM- CaCl_2 , gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and containing 5mM-glucose, 5mM-DL-3-hydroxybutyrate, 50mM-sucrose, 2% (v/v) MEM amino acid supplement (adjusted to pH 7.4 with NaOH) and 0.5g of bovine plasma albumin/100ml. The pieces were transferred to 5ml of basic medium containing trypsin (5mg) and incubated at 37°C for 10min with shaking (130 cycles/min) in

50ml screw-cap polythene bottles. After centrifugation for 1min at maximum speed in an MSE Super-Major centrifuge, the medium was aspirated and replaced with 5ml of basic medium lacking CaCl_2 and MgSO_4 and containing 2mM-EGTA* and trypsin inhibitor (1mg/ml). Incubation was continued with shaking for 5min. The medium was removed by centrifugation and the pellet washed twice by resuspension in 8ml of complete basic medium. The pellet was then resuspended in 5ml of basic medium containing 7.5mg of collagenase and incubated with shaking for approx. 35–45min (until the tissue was largely dispersed as judged by eye). Dispersion was completed by sucking up and down in a 2ml plastic pipette (tip bore approx. 0.5–1mm), and undigested material removed by filtration through nylon mesh. The cell suspension was layered on 5ml of basic medium containing 4g of bovine plasma albumin/100ml in a 10ml polypropylene centrifuge tube, and the cells were packed by centrifuging for 5min at 50g at room temperature (20°C). The cells were washed twice in the same medium by resuspension (plastic pipette) and centrifugation, and then resuspended in basic medium containing 2g of bovine plasma albumin/100ml to a concentration of 4×10^6 cells/ml. The preparation was assessed at this stage by light microscopy by exclusion of Trypan Blue (Tenant, 1964).

Preparation and incubation of parotid pieces. Parotid pieces were prepared by cutting rat parotid glands into pieces weighing 5–10mg with scissors in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing only 1mM- CaCl_2 , 5mM-glucose, 5mM-DL-3-hydroxybutyrate and gassed with $\text{O}_2 + \text{CO}_2$ (95:5). Incubations were made in the same medium with additions as given in the text, Tables and Figures.

Incubations and perfusion of parotid cells. For measurement of amylase release, spaces or metabolites, parotid cells were incubated in or perfused with bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 1mM- CaCl_2 , and with 5mM-glucose, 5mM-DL-3-hydroxybutyrate, 2% (v/v) MEM amino acid supplement, 50mM-sucrose, 2g of bovine plasma albumin/100ml and gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and with other additions as given in the text, Tables and Figures. Incubations were made in screw-cap polythene bottles at 37°C with shaking (60 cycles/min). The concentration of cells is given in the text, Tables and Figures. Perfusion of cells was achieved by embedding the cells in Sephadex G-10 or (where ^{45}Ca efflux was measured) in acetylated Sephadex G-10 (acetylation largely prevented Ca^{2+} adsorption to Sephadex). The perfusion chamber consisted of a 100 μl Oxford Sampler tip, sealed with cottonwool (or acetylated cotton-

wool) containing a layer of Sephadex (or acetylated Sephadex), 1.5cm in length. Parotid cells (2×10^6) were added by injection and sealed by addition of an upper column of Sephadex or acetylated Sephadex (0.25cm). The Sephadex G-10 used as a matrix for the cells was swollen in water at room temperature for 48h and equilibrated with perfusion medium before use. Acetylated cottonwool and acetylated Sephadex were washed with water for 30min (to remove ethanol) and equilibrated with perfusion medium before use. Perfusion was made with a peristaltic pump (Pharmacia P-3) at a flow rate of 0.2ml/min. The whole apparatus was assembled in a constant-temperature cabinet maintained at 37°C. Some preparations of parotid cells had high initial basal rates of amylase release, which declined to a low steady-state value after 1–2h. Therefore in all experiments the cells were preincubated or pre-perfused for 2h before incubation or perfusion.

In experiments with Ca^{2+} -free medium, bicarbonate-buffered saline (Krebs & Henseleit, 1932), without CaCl_2 and MgCl_2 but containing 2g of bovine plasma albumin/100ml, 50mM-sucrose, 5mM-glucose and 5mM-DL-3-hydroxybutyrate, was freed of traces of bivalent metal ions by passage through Chelex-100 resin processed according to the manufacturer's instructions and equilibrated with the medium. The medium was completed by addition of spectrographically standardized MgSO_4 .

Analytical methods. Citrate synthase was assayed spectrophotometrically in extracts of mitochondria or parotid pieces by a modification of the method of Srere *et al.* (1963), as given by Coore *et al.* (1971). Amylase was assayed in incubation or perfusion media or in extracts of parotid cells or parotid pieces by the method of Bernfeld (1955). Lactate dehydrogenase was assayed in incubation media or in extracts of parotid cells by the method of Bergmeyer & Brent (1974). ATP was assayed in HClO_4 extracts of parotid cells and pieces by the luciferin-luciferase assay (Stanley & Williams, 1969). DNA was assayed in parotid cells or parotid pieces by Burton's (1956) modification of the method of Schneider. Protein was assayed in extracts of parotid cells and pieces by the method of Gornall *et al.* (1949), and in mitochondria by the method of Lowry *et al.* (1951). Parotid cells were counted with light microscopy in a Neubauer haemocytometer. Ca^{2+} and Mg^{2+} were determined in parotid cells and pieces with an EEL 240 atomic absorption spectrophotometer as described by Severson *et al.* (1974). K^+ was also measured by atomic-absorption spectrophotometry after dilution with 20mM- LaCl_3 .

Extracts of mitochondria for assay of citrate synthase were prepared by alternate freezing (liquid N_2) and thawing (three times) in assay buffer (Coore *et al.*, 1971). For assay of lactate dehydrogenase and amylase, parotid pieces were disrupted in 20mM-

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid.

sodium phosphate, pH 6.9, with a Polytron PT-10 tissue homogenizer (30 s at position 4). For assay of citrate synthase the homogenate was frozen and thawed (three times). For assay of enzymes in parotid cells, the cells were disrupted in the incubation medium with a Polytron PT-10 homogenizer. For assay of ATP in parotid cells, HClO_4 was added to a final concentration of 5% (v/v). For assay of ATP in parotid pieces, the pieces were frozen in liquid N_2 , powdered in a mortar cooled in liquid N_2 , and ATP was extracted into 5% (v/v) HClO_4 . For assay of protein, parotid cells were freed of extracellular protein (albumin) by layering on protein-free medium containing 10% (w/v) Ficoll and centrifuged at 50g for 5 min. The supernatant was aspirated and the pellet resuspended in water.

Spaces. The intracellular water volume in parotid cells and pieces was estimated with $^3\text{H}_2\text{O}$ by using $[\text{U-}^{14}\text{C}]\text{sucrose}$ as an extracellular marker. Cells were incubated with media containing $^3\text{H}_2\text{O}$ ($2\mu\text{Ci/ml}$) and $[\text{U-}^{14}\text{C}]\text{sucrose}$ ($0.2\mu\text{Ci/ml}$) and subsequently separated by centrifuging for 20 s in an Eppendorf 3200 centrifuge. The supernatant was aspirated and retained for assay of radioactivity, and the pellet taken up in water and transferred quantitatively to methoxyethanol toluene-based scintillator (Severson *et al.*, 1974). Parotid pieces were blotted, weighed, and extracted with a Polytron PT-10 homogenizer, and the homogenates added to methoxyethanol toluene-based scintillator. For measurement of ^{45}Ca spaces, incubations were made with ^{45}Ca ($0.2\mu\text{Ci/ml}$) and $[6,6\text{-}^3\text{H}]\text{sucrose}$ ($2\mu\text{Ci/ml}$) by the same procedure. Radioactivity was assayed in media and extracts of parotid cells and pieces by dual-isotope liquid-scintillation spectrometry, and quench corrections were applied by means of an external standard.

Mitochondrial exchangeable Ca^{2+} . Parotid pieces were incubated in media containing ^{45}Ca ($1\mu\text{Ci/ml}$). At the end of incubation the pieces were rapidly collected by filtration through nylon mesh and disrupted in ice-cold medium [0.25M -sucrose/ 10mM -Tris/ HCl / 2mM -EGTA/ $5\mu\text{g}$ of Ruthenium Red/ ml (pH 7.4)] with a Polytron PT-10 homogenizer (two bursts of 2 s on position 3.5). The homogenate was centrifuged at 2°C for 10 min at 1000g and the supernatant removed and centrifuged at 2°C for 10 min at 10000g. The pellet was resuspended gently in the same medium (30 ml) and centrifuged for 10 min at 1000g and the supernatant centrifuged for 10 min at 10000g. The mitochondrial pellet was then taken up in $200\mu\text{l}$ of citrate synthase assay buffer and disrupted by freezing and thawing (see under 'Analytical methods'). One sample ($140\mu\text{l}$) was taken for assay of radioactivity, and citrate synthase was assayed in the remainder.

Citrate synthase was used as a mitochondrial marker enzyme to provide an index of the amount of mitochondria derived from individual batches of

parotid pieces. The mitochondrial exchangeable Ca^{2+} has been calculated as nmol of Ca^{2+} (on the basis of specific radioactivity of extracellular Ca^{2+}) per unit of citrate synthase.

Calculations. Units of enzyme activity are expressed as μmol of substrate utilized or product formed/min at 30°C .

Results and Discussion

Preparation, electron microscopy and some aspects of the composition and metabolic activities of rat parotid cells isolated by controlled proteolysis and incubated in vitro

Parotid cells have been prepared in this study by sequential incubations of parotid pieces with trypsin followed by collagenase. When this work was commenced Amsterdam & Jamieson (1973) had described preparation of pancreatic acinar cells, but it was found necessary to develop a different method for isolation of viable parotid cells. The method reported here has been very reproducible, and some 150 preparations have been successfully performed over a period of about 2 years. As judged by exclusion of Trypan Blue under the light microscope, at least 95% of cells were viable in all preparations used in this study.

Plate 1 shows an electron micrograph of a parotid cell prepared by this technique and typical of many examined in a single preparation. The cell shows a sharply defined boundary and intact plasma membrane with nucleus on one side of the cell, granules to the opposite side, microvilli, mitochondria with well-preserved cristae, sharply defined rough endoplasmic reticulum, secretory granules and a large nucleus with well-preserved nuclear membrane. Electron microscopy showed that at least 90% of cells in this particular preparation contained zymogen.

Table 1 shows some aspects of the composition of parotid cells and parotid pieces. Protein concentrations were very similar, whereas the intracellular water was higher in cells than in pieces. This does not necessarily indicate hydration of cells, as protein determinations, which formed the basis for comparison, may include extracellular proteins in the case of parotid pieces. This suggestion is perhaps supported by the observation that the DNA concentration in cells was higher than that in pieces; the intracellular water content, in $\mu\text{l/mg}$ of DNA, was 70 for cells and 94 for pieces. The amylase concentrations were similar on a unit weight basis, but on a DNA basis the cell concentration was only 61% of that of pieces, suggesting loss of the enzyme during cell preparation. The concentration of ATP, though higher in cells on a unit weight basis, was almost identical in cells and pieces on a DNA basis. Concentrations of Ca^{2+} , Mg^{2+} and K^+ were similar on a unit weight basis, but on a DNA basis the cell concentration was only

EXPLANATION OF PLATE I

Electron micrograph of a typical parotid cell

Parotid cells were prepared as described in the Experimental section, and packed by centrifuging for 30s in an Eppendorff 3200 centrifuge. The supernatant was aspirated, and the cell pellet removed and fixed in 2.5% (w/v) glutaraldehyde in 0.1M-sodium phosphate buffer, pH7.3, for 18h. The pellet was then exposed to 1% OsO₄ in 0.2M-sodium phosphate, pH7.3, for 1h and washed and dehydrated with aq. 70% (v/v) ethanol. The pellet was embedded in Araldite, dried for 3 days at 55–60°C, and sectioned in an LKB Ultratome. The sections were stained with 2% (w/v) uranyl acetate in methanol and then in Renolds lead citrate, and examined in a Phillips 300 electron microscope. Magnification $\times 10087$. P, plasma membrane; M, mitochondrion; R, rough endoplasmic reticulum; S, secretory granule; V, microvillus. N, nucleus.

Table 1. Some aspects of the composition of rat parotid cells and parotid pieces

Rat parotid cells and parotid pieces were prepared as described in the Experimental section and incubated at 37°C for either 2h (cells) or 30min (pieces) in bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 1mM-CaCl₂, gassed with O₂+CO₂ (95:5) and containing 5mM-glucose, 5mM-DL-3-hydroxybutyrate and (for cells only) 20mg of bovine plasma albumin/ml, 50mM-sucrose and 0.02vol. of MEM amino acid supplement. For details of estimations, see the Experimental section. The values given are for a minimum of six observations, except for concentrations of Ca²⁺, Mg²⁺ and K⁺ in pieces, which are based on three observations. The values are given in terms of mg dry wt. of cells or tissue, although, except for the metals, they were measured in terms of mg of protein. Conversion was effected by use of values for protein concentration given in the first line of the Table. Values are means (±s.e.m. in some cases).

Parameter	Parotid cells	Parotid pieces
Protein concn. (mg of protein/mg dry wt.)	0.77±0.05	0.77±0.02
Cell count (number of cells/mg dry wt.)	1.99×10 ⁶ ±9×10 ⁴	—
Protein content per cell (ng)	0.39	—
Average cell dry wt. (ng)	0.50	—
Intracellular water (μl/mg dry wt.)	2.03±0.10	1.6±0.06
Average cell water content (ng)	1.02	—
Average cell wet wt. (ng)	1.52	—
Amylase (units/mg dry wt.)	234±13.9	226±7.7
DNA (mg/mg dry wt.)	0.03±0.0023	0.017±0.007
Ca ²⁺ } (ng-atoms/mg dry wt.)	21±1.2	17±1.0
Mg ²⁺ }	30±0.8	26±1.3
K ⁺ }	262±4	237±8
ATP (nmol/mg dry wt.)	6.1±0.1	3.6±0.5
Citrate synthase (units/mg dry wt.)	—	0.039±0.026
Citrate synthase (units/mg of mitochondrial protein)	—	0.47±0.035
Mitochondrial protein (mg/mg dry wt.)	—	0.08

Fig. 1. Effects of dimethyl sulphoxide, ionophore A23187 in dimethyl sulphoxide and high-K⁺ medium on amylase release by perfused parotid cells

Parotid cells were prepared, pre-perfused and perfused as described in Table 2. Δ, Perfusion switched to medium containing dimethyl sulphoxide (2 μl/ml) at point A and to medium containing 40 μM-ionophore A23187 and dimethyl sulphoxide (2 μl/ml) at point B. ▲, Perfusion switched to medium in which 95 mmol of NaCl/l was replaced by KCl (final concn. of KCl, 100mM) at point B.

Fig. 2. Effects of adrenaline (10 μM) and carbamoylcholine (1 μM) on the release of amylase by rat parotid cells in the presence or absence of external Ca²⁺

Parotid cells were prepared, pre-perfused and perfused as described in Table 3. ●, Complete medium switched to 1 μM-carbamoylcholine at A; ○, Ca²⁺-free medium switched to 1 μM-carbamoyl choline at A; ▲, complete medium switched to 10 μM-adrenaline at A; Δ, Ca²⁺-free medium switched to 10 μM-adrenaline at A.

Table 2. *Effects of adrenaline, isoproterenol, carbamoylcholine, 3-isobutyl-1-methylxanthine, dibutyryl cyclic AMP, ionophore A23187 and high K⁺ concentration on amylase release*

Parotid cells were perfused at 37°C with bicarbonate-buffered saline containing albumin, sucrose, glucose, 3-hydroxybutyrate and amino acids (for details of preparation of cells and of apparatus see under 'Methods'; for composition of the medium see Table 1). After 2 h of pre-perfusion, release of amylase was followed by assay of the enzyme in fractions of perfusate collected at 2 min intervals. The control rate of release was measured during 0–20 min of perfusion, and the rate of release with the substances shown measured over a further 20–30 min of perfusion. It was shown in separate experiments that the control rate is constant to within 10% for at least 60 min of perfusion. The initial rate of release with the substances shown (column 3, experimental) was the maximum rate of release after their introduction, and this occurred within the first 4 min. The duration of the increased rate of release is shown in column 4. Ionophore A23187 was added in dimethyl sulphoxide (2 µl/ml); control experiments showed that dimethyl sulphoxide had little, if any, effect on the rate of amylase release. Other substances were dissolved directly into the perfusion medium. In high-K⁺ perfusions, the concentration of K⁺ was raised from 5 to 100 mequiv./l by substitution of KCl for NaCl.

Experiment	Initial rate of amylase release (units of amylase/2 min per mg of cell protein)		Duration of stimulated amylase release (min)
	Control	Experimental	
Adrenaline (10 µM)	0.69	2.66	>20
(1 µM)	0.62	1.36	>20
(0.1 µM)	0.58	1.10	>20
Isoproterenol (10 µM)	0.64	1.90	>20
Carbamoylcholine (1 µM)	0.62	1.53	>20
(0.1 µM)	0.62	1.19	8
3-Isobutyl-1-methylxanthine (1 mM)	0.64	1.64	>20
Dibutyryl cyclic AMP (2 mM)	0.64	0.92	>20
Ionophore A23187 (40 µM)	0.78	3.34	>20
(5 µM)	0.78	2.80	>10
High-K ⁺ (100 mM)	0.64	1.30	4

Table 3. *Effects of adrenaline, isoproterenol, phenylephrine and carbamoylcholine on amylase release by parotid cells or parotid pieces in the presence and in the absence of external Ca²⁺*

For details of preparation of parotid cells and the perfusion apparatus see the Experimental section. Parotid cells (2×10^6) were pre-perfused for 2 h at 37°C with bicarbonate-buffered saline containing albumin, sucrose, glucose, 3-hydroxybutyrate and amino acids (for composition see Table 1), and for a further 20 min with the same medium or with similar medium without Ca²⁺ (see the Experimental section). Control experiments with ⁴⁵Ca in the absence of cells showed that 20 min of perfusion with Ca²⁺-free medium removed not less than 84% of Ca²⁺ from the perfusion cell. Perfusion was then continued with collections for assay of amylase, for measurement of control rates of release and initial rates of release with hormonal stimulation as given in Table 2. Parotid pieces were prepared as described in the Experimental section and preincubated for 30 min at 37°C (50 mg in 3 ml) in bicarbonate-buffered medium (Krebs & Henseleit, 1932), modified to contain 1 mM-CaCl₂ and containing 5 mM-D-glucose, 5 mM-DL-3-hydroxybutyrate and gassed with O₂ + CO₂ (95:5). Preincubation was continued for 20 min in 5 ml of the same medium or for 2 × 10 min in 5 ml of similar medium without Ca²⁺ (see the Experimental section) and containing 0.2 mM-EGTA. Amylase release was then measured during a subsequent 30 min incubation in fresh medium of the same composition and with additions shown in column 1 for experimental values. Results for cells are based on single perfusions. Results for pieces are means of three observations (±S.E.M.). **P* < 0.01 (control versus experimental). For other differences *P* > 0.05.

Experiment	External Ca ²⁺	Initial rate of amylase release by parotid cells (units/2 min per mg of cell protein)		Amylase release by parotid pieces (units/30 min per mg of cell protein)	
		Control	Experimental	Control	Experimental
Adrenaline (1 µM)	+	0.50	2.25	9.44 ± 1.0	35.8 ± 0.10*
	—	0.70	2.50	8.14 ± 3.1	30.2 ± 0.44*
Isoproterenol (1 µM)	+	0.64	1.90	9.44 ± 1.0	37.2 ± 0.15*
	—	0.63	1.42	8.14 ± 3.1	28.5 ± 4.4*
Phenylephrine (1 µM)	+	—	—	9.44 ± 1.0	18.4 ± 1.7*
	—	—	—	8.14 ± 3.1	10.6 ± 0.9
Carbamoylcholine (1 µM)	+	0.53	1.31	9.44 ± 1.0	29.2 ± 5.3*
	—	0.97	1.05	8.14 ± 3.1	13.4 ± 1.9

about 65% of that of pieces. Whether this reflects losses during preparation or association of these ions with extracellular structures is not known.

Rates of amylase release by cells in response to a number of secretagogues are shown in Tables 2 and 3 and Figs. 1 and 2. The highest rate of release with adrenaline (2.8 units of amylase/2 min per mg of protein) corresponds to the release of 36% of the stored enzyme per h. In some experiments the rate of release of lactate dehydrogenase from cells was measured during batch incubation. Basal release was measured over 4 h and with adrenaline for 1 h. Basal release of lactate dehydrogenase was linear and corresponded to 4% of the cell content of this enzyme per h. Adrenaline had no effect on lactate dehydrogenase release; adrenaline did, however, stimulate amylase release (see below), and, moreover, effects of adrenaline were abolished by uncouplers of oxidative phosphorylation (0.5 mM-2,4-dinitrophenol or 50 μ M-carbonyl cyanide trifluoromethoxyphenylhydrazine; results not shown). This suggests that release of amylase is occurring, not through breakage of a proportion of cells, but rather through a specific process of amylase secretion, perhaps requiring ATP. The data do not permit comparison of rates of amylase release by cells and pieces to be made under strictly comparable conditions, as cells were perfused and pieces were incubated in batches in a fixed volume of medium. Under these rather different conditions rates of amylase release by cells and pieces were very similar.

As judged by Trypan-Blue exclusion, electron microscopy, protein, water, electrolyte and ATP content and amylase and lactate dehydrogenase release the preparations of cells appear to be viable in terms of morphological and functional criteria.

Mangos *et al.* (1975a,b,c) have described the preparation of free parotid cells, by digestion with collagenase, hyaluronidase and trypsin and disruption by pipetting, and stimulation of amylase release by β -adrenergic effectors, dibutyryl cyclic AMP and cholinergic effectors. The proportion of zymogen-containing cells in the preparation (95%) and the secretory responses to adrenaline, isoproterenol and dibutyryl cyclic AMP were similar. The threshold sensitivity to adrenaline may be similar, although the studies by Mangos *et al.* (1975b) encompassed a wider range of concentration. The maximum rate of amylase release observed by Mangos *et al.* (1975a,b), which was similar to rates of release *in vivo*, was somewhat higher than in the present study. We suggest that two factors leading to depletion of cell amylase may be responsible for lower rates of release in this preparation. One is the use of fed rats and the other is the loss of amylase during cell preparation referred to above. K^+ concentration in parotid cells was 129 mequiv./l in the present study as opposed to 80 mequiv./l in the cells used by Mangos *et al.* (1975a).

Effects of α - and β -adrenergic effectors, cholinergic effectors, 3-isobutyl-1-methylxanthine, dibutyryl cyclic AMP, ionophore A23187 and high K^+ concentration on amylase release by rat parotid cells and parotid pieces

The purpose of these experiments was to define the secretory responses of parotid cells with respect to amylase release. Perfusion was chosen as the means of study, because it allows continuous monitoring of release rate, continuous removal of released amylase and metabolites, and rapid introduction or removal of substances to be tested. This has necessitated development of a technique of perfusion suitable for use with a dispersed cell preparation (see the Experimental section). With the conditions used, the basal rate of release of amylase was approx. 8% of the cell content/h and was constant for at least 2 h of perfusion (the longest time-period studied). Therefore it has been possible to study effects of various potential secretagogues on the rate of amylase release without the need for parallel control perfusions. Fig. 1 shows the results of two typical experiments with this technique. The examples shown are ionophore A23187, a bivalent-metal ionophore (Reed & Lardy, 1972), which induced a prompt and fourfold increase in the rate of amylase release which lasted for approx. 8–10 min before falling to a lower sustained and increased rate. This effect was not seen with dimethyl sulphoxide (the solvent in which ionophore A23187 was introduced). Fig. 1 also shows that high K^+ concentration produces only a small short-lived increase in amylase release.

The secretory responses of perfused parotid cells to various effectors have been summarized in terms of the maximum (initial) rate of amylase release (Tables 2 and 3) and the duration of the stimulated release (Table 2). Amylase release by parotid pieces during 30 min of batch incubation is shown in Table 3.

Amylase release by parotid cells and by parotid pieces was shown to be enhanced by adrenaline, isoproterenol, carbamoylcholine and phenylephrine (only tested in pieces). These findings indicate stimulation of amylase release through α -adrenergic receptors (phenylephrine), β -adrenergic receptors (isoproterenol) and cholinergic receptors (carbamoylcholine). In cells, responses were shown to be concentration-dependent over the range 0.1–100 μ M for adrenaline and isoproterenol and 0.1–1 μ M for carbamoylcholine (full range not shown in Table 2). Amylase release from cells was also stimulated by 3-isobutyl-1-methylxanthine (1 mM), an inhibitor of cyclic AMP phosphodiesterase, and by dibutyryl cyclic AMP. The order of effectiveness of these agents in terms of total amylase release (results not shown) was isoproterenol and 3-isobutyl-1-methylxanthine > adrenaline > phenylephrine, carbamoylcholine and dibutyryl cyclic AMP. Amylase release from cells was

also stimulated by the bivalent-cation ionophore A23187; the effect of ionophore A23187 was largely abolished by omission of CaCl_2 from the perfusion medium (not shown). This might suggest that entry of Ca^{2+} induced by the ionophore can lead to amylase release. High extracellular K^+ concentration produced only a small and transient increase. Except for the effects of high K^+ concentration and of $0.1 \mu\text{M}$ -carbamoylcholine, stimulation of amylase release was sustained for at least 20 min (this was the maximum period of measurement of amylase release after introduction of the stimulus).

Table 3 and Fig. 2 show the dependence of secretory responses on extracellular Ca^{2+} in parotid cells and parotid pieces. In cells or pieces (Table 3 and Fig. 1), the release of amylase in response to adrenaline or isoproterenol was not dependent on extracellular Ca^{2+} , whereas release in response to carbamoylcholine was abolished in the absence of extracellular Ca^{2+} . In pieces, the response to phenylephrine was also lost in the absence of extracellular Ca^{2+} . Thus β -adrenergic effects on release are retained whereas α -adrenergic and cholinergic effects on release are lost in the absence of extracellular Ca^{2+} . It may be noted that the basal rate of release of amylase by cells tended to be higher in a Ca^{2+} -free medium.

The efficacy of procedures for depleting extracellular Ca^{2+} with cells was ascertained with ^{45}Ca and leads to removal of at least 94% of extracellular Ca^{2+} from the perfusion cell. With pieces, control experiments with $[^3\text{H}]\text{sucrose}$ showed that the wash procedure removed at least 85% of the extracellular marker; the removal of extracellular Ca^{2+} may have been more extensive because of the inclusion of EGTA in the wash medium.

Incorporation of ^{45}Ca into rat parotid cells, rat parotid pieces and the mitochondrial fraction of rat parotid pieces; validity of the procedures

The procedures developed for measurement of the incorporation of ^{45}Ca into rat parotid cells and parotid pieces and into the mitochondrial fraction of parotid pieces are similar in principle to those used for parallel studies with rat epididymal fat-pads and fat-cells (Severson *et al.*, 1976; Randle *et al.*, 1974).

Parotid cells and pieces. For determination of ^{45}Ca incorporation into parotid cells and parotid pieces, incubations were made in medium containing ^{45}Ca and also $[6,6\text{'-}^3\text{H}]\text{sucrose}$ as an extracellular marker. Parotid cells and parotid pieces were separated from incubation medium by centrifugation and aspiration of medium (cells) or by removal and blotting (pieces). Extracts of cells and pieces, and incubation media were then assayed for ^{45}Ca and $[6,6\text{'-}^3\text{H}]\text{sucrose}$ by dual radioisotope counting. The incorporation of ^{45}Ca was then corrected for extracellular ^{45}Ca in incubation medium included in the cell pellet or in

incubation medium and in extracellular fluid in parotid pieces by use of sucrose as an extracellular marker. The calculation assumes, for pieces, that the concentration of ^{45}Ca in the sucrose space of the tissue is the same as that in the incubation medium. The sucrose space of the cell pellet was approx. $3.1 \mu\text{l}/\text{mg}$ dry wt. of cells and that of the parotid pieces was approx. $1.8 \mu\text{l}/\text{mg}$ dry wt. of tissue. The corrections to be applied for extracellular ^{45}Ca amounted to 63 and 32% of the total ^{45}Ca incorporated into the cell pellet and pieces respectively.

Mitochondrial fraction. To determine incorporation of ^{45}Ca into the mitochondrial fraction, rat parotid pieces were disrupted with a Polytron homogenizer into extraction medium within 10–15 s of the end of incubation. It was necessary to ensure that redistribution of ^{45}Ca between mitochondrial and extramitochondrial fractions did not take place during separation and purification of the mitochondrial fraction. This was accomplished by inclusion of Ruthenium Red ($5 \mu\text{g}/\text{ml}$) and EGTA (2 mM) in the medium used to extract, isolate and purify the mitochondrial fraction. Parotid mitochondria, like mitochondria prepared from other rat tissues, possess a very active Ca^{2+} -uptake system, which may be driven by either respiration (blocked by uncouplers) or ATP (blocked by oligomycin) (Dormer & Ashcroft, 1974). This uptake system is effectively inhibited by Ruthenium Red at the concentration used ($5 \mu\text{g}/\text{ml}$). The inclusion of EGTA results in sequestration of extramitochondrial Ca^{2+} .

The efficacy of the combined action of Ruthenium Red and EGTA was shown in experiments in which parotid pieces were incubated in the absence of ^{45}Ca and disrupted in extraction fluid containing ^{45}Ca and with or without Ruthenium Red and EGTA. In the absence of Ruthenium Red and EGTA the mitochondrial incorporation of ^{45}Ca during tissue disruption and isolation and purification of mitochondria was 3800 d.p.m./unit of citrate synthase; this was decreased by inclusion of Ruthenium Red and EGTA to 120 d.p.m./unit of citrate synthase. Control experiments, in which $[6,6\text{'-}^3\text{H}]\text{sucrose}$ was included as an extracellular marker, showed that the final mitochondrial pellet was free of $[^3\text{H}]\text{sucrose}$ and it is assumed that extracellular ^{45}Ca was also removed by the fractionation procedure. As in the study with rat epididymal adipose tissue (Severson *et al.*, 1976), the major factor preventing incorporation of ^{45}Ca into the mitochondrial fraction after tissue disruption and during isolation and purification of mitochondria was EGTA (results not shown). Mitochondrial fractions were prepared, in extraction medium with Ruthenium Red and EGTA, from parotid pieces incubated with ^{45}Ca ; they were then incubated at 0°C in the same extraction medium. The loss of ^{45}Ca from the mitochondrial fraction was less than 6% after 60 min. These studies appear to show

that little or no redistribution of ^{45}Ca is to be expected during the procedure used for isolation and purification of the mitochondrial fraction.

The yield of purified mitochondria (on the basis of citrate synthase assays) was approx. 20–30%, and, because of the low yield, ^{45}Ca incorporations into mitochondrial fractions have been calculated and compared as nmol of medium ^{45}Ca incorporated/unit of citrate synthase. The data given in Table 1 would permit approximate calculation of incorporations/mg of dry cells, per mg of cell protein or per mg of mitochondrial protein. Parotid zymogen granules can be a contaminant of mitochondrial fractions, and such granules contain Ca^{2+} but apparently do not incorporate ^{45}Ca (Feinstein & Schramm, 1970). The procedure used to isolate and purify the mitochondrial fraction in the present study resulted in only small contamination with secretory granules. This was ascertained by assay of amylase in the purified mitochondrial fraction after freezing and thawing and taking up in citrate synthase assay buffer (see the Experimental section). The amylase activity of the mitochondrial fraction was less than 1% of that of the tissue. Finally, it may be noted that mitochondria isolated by this technique exhibited good respiratory control; the respiratory-control ratios (Chance & Williams, 1955) were 4.5–9.1 with 5 mM-2-oxoglutarate, 0.5 mM-L-malate and 1 μmol of ADP (in 4 ml).

Incorporation of ^{45}Ca into parotid cells and parotid pieces

As shown in Fig. 3, there was an extremely rapid initial incorporation of ^{45}Ca into parotid cells, which was substantially complete within 1–2 min. The extent of this incorporation (equivalent to 2.85 ng-atoms of medium Ca^{2+} /mg dry wt.) during the first min of incubation corresponded to 14% of total cell Ca^{2+} (cf. data of Table 1). Thereafter incorporation of ^{45}Ca proceeded at a very low and constant rate, which was only apparent on more prolonged incubation (3 h; results not shown). This slow phase of incorporation was at a rate of 0.19 ng-atom of medium Ca^{2+} /h per mg dry wt. (i.e. no more than 0.11% of the initial rate). This may show that there is a very rapidly exchanging pool of Ca^{2+} of modest dimensions in parotid cells, and that the bulk of cell Ca^{2+} does not exchange with extracellular Ca^{2+} at any appreciable rate. Incorporation of ^{45}Ca into parotid pieces was substantially slower and only achieved equilibrium after 30 min of incubation (Fig. 3). The extent of incorporation (i.e. after 30 min) corresponded to 4.43 ng-atoms of medium Ca^{2+} /mg dry wt. of tissue or 26% of total tissue Ca^{2+} . The extent of Ca^{2+} incorporation into pieces is thus approximately twice as great as in cells, though occurring at a lower rate. The reason for these findings cannot be ascertained from the data. The more extensive incorporation in

Fig. 3. Incorporation of ^{45}Ca into parotid cells, parotid pieces and the mitochondrial fraction of parotid pieces at different times of incubation.

The incorporations of ^{45}Ca into parotid cells and parotid pieces were measured as described in Table 4, except that the times of incubation were as shown in the Figure. Incorporations of ^{45}Ca into the mitochondrial fraction of parotid pieces were measured as described in Table 5, except that incubations were for the times shown. There were at least three observations for each point. ●, Parotid cells; ○, parotid pieces; ▲, mitochondrial fraction from parotid pieces. Standard errors are shown.

pieces could be due to an additional but more slowly exchanging pool in structures other than parotid zymogen-containing cells. Slower equilibration in pieces suggests the possibility that extracellular diffusion of ^{45}Ca limits the rate of exchange with cell Ca^{2+} . The slow equilibration of the extracellular compartment in parotid pieces is suggested by experiments with ^3H sucrose, in which complete equilibration of the extracellular compartment required 20 min of incubation (results not shown).

Incorporation of ^{45}Ca into mitochondrial fraction of parotid pieces

Fig. 3 shows the incorporation of ^{45}Ca into the mitochondrial fraction of parotid pieces during 60 min of incorporation. Incorporation into this fraction was more complex, showing an initial and rapid incorporation during the first 2 min followed by a lower and steady rate of incorporation from 2 to 60 min. Incorporation during the first 2 min was 2.9 ng-atoms of medium Ca^{2+} /unit of citrate synthase, corresponding to 1.4 ng-atoms of medium Ca^{2+} /mg of mitochondrial protein or 0.11 ng-atom of medium Ca^{2+} /mg dry wt. of tissue. Incorporations after 60 min of incubation were approximately twice those after 2 min of incubation. Mitochondrial total Ca^{2+} was not measured in these experiments because of the

In additional experiments (not shown) the time-course of ^{45}Ca incorporation with adrenaline and

Fig. 4. Effect of adrenaline concentration and carbamoylcholine concentration on the incorporation of ^{45}Ca into rat parotid cells

The incorporations of ^{45}Ca into parotid cells were measured over 1 h of incubation by using the conditions and methods described in Table 4. There were three observations for each point for adrenaline (●) and single observations for each point with carbamoylcholine (○). Standard errors are shown for adrenaline.

carbamoylcholine was investigated. It was not possible to detect an effect of these agents on the initial rapid incorporation of ^{45}Ca (i.e. in the first 2 min). The time involved in separating samples for analysis did not permit measurements over very short periods of incubation. Adrenaline and carbamoylcholine had marked stimulatory effects on incorporation in the slow phase, which persisted for approx. 2 h. Thereafter the rate of incorporation declined and fell below the control value after 2 h (although the absolute incorporation was still increased). Whether this increase in ^{45}Ca represents equilibration of a slowly exchanging pool of finite size (approx. 2.3 ng-atoms of medium Ca^{2+} /mg dry wt. or approx. 10% of total cell Ca^{2+}) or uptake of additional Ca^{2+} or both is not known. Incorporation of ^{45}Ca into parotid cells was also enhanced by Ruthenium Red (5 $\mu\text{g}/\text{ml}$) (results not shown).

Effects of adrenergic and cholinergic effectors, dibutyryl cyclic AMP and 2,4-dinitrophenol on incorporation of ^{45}Ca into mitochondrial fraction of parotid pieces

Columns 1 and 2 of Table 5 show effects of various agents on the incorporation of ^{45}Ca into the mitochondrial fraction of parotid pieces during 10 min of incubation (incubations in Table 4 were for 60 min).

Table 5. Effects of adrenaline, isoproterenol, phenylephrine, carbamoylcholine, dibutyryl cyclic AMP and 2,4-dinitrophenol on ^{45}Ca incorporated into mitochondria of rat parotid pieces

Parotid pieces were prepared (see the Experimental section) and preincubated for 30 min (pieces from one gland in 5 ml) in bicarbonate-buffered saline, containing glucose and DL-3-hydroxybutyrate (for composition see Table 1). They were then incubated for 10 min (or 60 min**) in 4 ml of similar medium containing ^{45}Ca (2 $\mu\text{Ci}/\text{ml}$) and with additions as shown. At the end of the incubation, mitochondria were isolated, extracted and assayed for citrate synthase and ^{45}Ca as described in the Experimental section. In experiments with Ca^{2+} -free medium, parotid pieces were incubated in medium containing ^{45}Ca (2 $\mu\text{Ci}/\text{ml}$) for 1 h. They were then washed by two 5 min incubations in Ca^{2+} -free medium and incubated for 10 min in Ca^{2+} -free medium with additions as shown. At the end of the incubation, mitochondria were isolated, extracted and assayed for citrate synthase and ^{45}Ca . Because of the number of samples, the second wash period varied from 5 to 9 min, although it was constant for each pair (control and experimental). The results in these experiments (column 4) have therefore been expressed by the change in ^{45}Ca (experimental - control) as a percentage of the control. Values are means $\pm$ S.E.M. of the numbers of observations given in parentheses. * $P < 0.01$, † $P < 0.05$ (experimental versus control) for columns 2 and 3. * $P < 0.01$ for difference from zero for column 4.

Experiment	Parotid mitochondrial ^{45}Ca		
	Ca^{2+} medium		Ca^{2+} -free medium ($\frac{\text{experimental} - \text{control}}{\text{control}} \times 100$)
	(ng-atoms of ^{45}Ca per unit of citrate synthase)		
	Control	Experimental	
Adrenaline (1 μM)	2.77 ± 0.21	3.18 ± 0.13 (6)	$-46.7 \pm 5.7^*$ (4)
Adrenaline (1 μM)**	4.41 ± 0.50	$7.38 \pm 0.35^*$ (4)	—
Isoproterenol (1 μM)	3.83 ± 0.17	$2.57 \pm 0.06^*$ (6)	$-38.4 \pm 9.3^*$ (4)
Phenylephrine (1 μM)	2.97 ± 0.14	3.17 ± 0.16 (6)	-5.5 ± 9.6 (4)
Phenylephrine (1 μM)**	4.41 ± 0.50	5.21 ± 0.30 (4)	—
Carbamoylcholine (1 μM)	2.77 ± 0.09	2.54 ± 0.07 (4)	-11.6 ± 5.5 (4)
Dibutyryl cyclic AMP (2 mM)	4.05 ± 0.14	$2.34 \pm 0.48^\dagger$ (4)	—
2,4-Dinitrophenol (0.5 mM)**	6.44 ± 0.46	$4.02 \pm 0.26^*$ (4)	$-39.0 \pm 7.6^*$ (4)

Adrenaline ($1\mu\text{M}$), phenylephrine ($1\mu\text{M}$) and carbamoylcholine ($1\mu\text{M}$) had no effect on ^{45}Ca incorporation into the mitochondrial fraction. Isoproterenol ($1\mu\text{M}$) and dibutyl cyclic AMP (2mM) substantially decreased the incorporation of ^{45}Ca into mitochondria. This might suggest that β -adrenergic effectors, acting perhaps via cyclic AMP, lower mitochondrial exchangeable Ca^{2+} in parotid. Table 5 also shows effects of adrenaline and 2,4-dinitrophenol after 60 min incubation. With the longer incubation period, adrenaline ($1\mu\text{M}$) significantly increased mitochondrial incorporation of ^{45}Ca , in conformity with the observations of Dormer & Ashcroft (1974). Mitochondrial exchangeable Ca^{2+} was decreased by 2,4-dinitrophenol over 60 min of incubation in spite of the fact that 2,4-dinitrophenol substantially increased exchangeable Ca^{2+} in the whole tissue (cf. Tables 4 and 5). These effects of 2,4-dinitrophenol may be compared with those seen in rat epididymal adipocytes, where the uncoupler decreases ^{45}Ca incorporation into mitochondria, but increases incorporation into the supernatant fraction. In adipocytes, 2,4-dinitrophenol did not increase total cell ^{45}Ca , but in that tissue (unlike parotid) the major part of ^{45}Ca incorporation is mitochondrial, the intracellular water volume is very small, and the decreased mitochondrial incorporation is apparently balanced by an increased incorporation into the supernatant (Severson *et al.*, 1976).

β -Adrenergic effectors failed to alter the total incorporation of ^{45}Ca into parotid pieces (or cells), but they diminished incorporation into the mitochondrial fraction of parotid pieces. The question arises whether the decreased incorporation into the mitochondrial fraction was balanced by increased incorporation into extramitochondrial fraction(s). Incorporation of ^{45}Ca into the mitochondrial fraction was only approx. 5% of the total incorporation and the decrease in incorporation with β -adrenergic effectors was only approx. 2% of the total incorporation. A change of this magnitude would be difficult to establish with the present technique.

Efflux of ^{45}Ca from parotid cells during perfusion with Ca^{2+} -free medium

Efflux of ^{45}Ca from parotid cells in a Ca^{2+} -free medium was investigated by perfusion of cells embedded in acetylated Sephadex G-10 and supported by a plug of acetylated cottonwool. Acetylation of the supporting matrix was essential to minimize binding of ^{45}Ca by the matrix. The acetylated materials can become deacetylated on storage, and it was found desirable to use them within 7 days of preparation. The result of an experiment with an aged preparation of acetylated Sephadex and cottonwool is shown in Fig. 5(c). By comparison with Figs. 5(a) and 5(b), in which fresh material was used, the release of ^{45}Ca was retarded.

Fig. 5. Effect of (a) 2 mM-dibutyl cyclic AMP, (b) 10 μM -adrenaline and (c) 10 μM -isoproterenol on ^{45}Ca efflux from rat parotid cells during perfusion with Ca^{2+} -free medium

For details of preparation of parotid cells and of perfusion apparatus, see the Experimental section. Parotid cells (8×10^6) were pre-perfused for 60 min at 37°C with bicarbonate-buffered saline, containing albumin, sucrose, glucose, 3-hydroxybutyrate and amino acids (for composition see Table 1). Pre-perfusion was then continued for a further 60 min with similar medium containing ^{45}Ca [$2\mu\text{Ci}/\mu\text{g-atom}$ per ml in (a) and (b) and $1\mu\text{Ci}/\mu\text{g-atom}$ per ml in (c)]. Perfusion was then commenced with a similar medium, without Ca^{2+} (see the Experimental section), and fractions were collected at 1 min intervals and assayed for radioactivity in ^{45}Ca (see the Experimental section). (a) 2 mM-Dibutyl cyclic AMP introduced at A; (b) 10 μM -adrenaline introduced at A; (c) 10 μM -isoproterenol introduced at A.

Equilibration of the column and cells with ^{45}Ca was achieved by 60 min of pre-perfusion with medium containing ^{45}Ca (1 or $2\mu\text{Ci}/\mu\text{g-atom}$ of Ca^{2+}/ml). Perfusion with Ca^{2+} -free medium was then commenced, and the perfusate collected as 1 min fractions and assayed for ^{45}Ca . With freshly acetylated matrix materials the efflux of ^{45}Ca from the column in the absence of cells was exponential according to the equation:

$$y = ae^{-bx}$$

where y is the fractional efflux of ^{45}Ca , x is the time in min and $a = 2.08 \pm 0.261$ and $b = -1.39 \pm 0.15$ (means $\pm$ S.E.M.).

In practical terms the efflux of ^{45}Ca fell by 10 min of perfusion to less than 1% of the total efflux per min in the absence of cells. The rate in the presence of cells (8×10^6 cells) was approximately twice the control rate after 10 min, and it is assumed that this increased rate is due to ^{45}Ca released by the parotid cells. On the basis of data given in Fig. 3, 8×10^6 cells

(approx. 4 mg dry wt.) incorporate 11.5 ng-atoms of ^{45}Ca , which corresponds to some 50000 d.p.m. at a medium specific radioactivity of $2\mu\text{Ci}/\mu\text{g-atom}$. This level of incorporation may be sufficient to account for the sustained and increased release of ^{45}Ca from perfusion columns containing cells as illustrated in Fig. 6 (the increased release from 10 to 40 min of perfusion was approx. 25000 d.p.m.). Rigorous mathematical analysis of the efflux due to the presence of cells was not possible, because efflux of ^{45}Ca from the matrix obscured efflux of ^{45}Ca from cells during the early period of perfusion. Incorporation of ^{45}Ca into the matrix was at least 440000 d.p.m. (i.e. at least ten times the incorporation into parotid cells). Further evidence that the higher and sustained rate of ^{45}Ca efflux in the presence of cells represents release of ^{45}Ca incorporated into cells has come from experiments with the bivalent-metal ionophore A23187 (results not shown). Addition of $40\mu\text{M}$ -ionophore A23187 after 28 min of perfusion with Ca^{2+} -free medium led to an immediate and very rapid rate of ^{45}Ca efflux of six to eight times the control rate, which was sustained for about 3 min. This increased rate of efflux was followed by a sharp decrease in the rate to less than 40% of the control rate. The additional ^{45}Ca released was equivalent to 1.06 ng-atoms of medium Ca^{2+} incorporated per mg dry wt. of cells. On the basis of the data given in Fig. 3, this would correspond to 37% of ^{45}Ca incorporated before perfusion with Ca^{2+} -free medium.

Fig. 6. Effect of (a) $1\mu\text{M}$ -carbamoylcholine and (b) $1\mu\text{M}$ -phenylephrine on ^{45}Ca efflux from rat parotid cells during perfusion with Ca^{2+} -free medium

For conditions of pre-perfusion and perfusion, see Fig. 5. (a) Carbamoylcholine ($1\mu\text{M}$) added during period shown by arrows; (b) $1\mu\text{M}$ -phenylephrine added during period shown by arrows. ●, Perfusion without cells; ▲, (a) $1\mu\text{M}$ -carbamoylcholine, (b) $1\mu\text{M}$ -phenylephrine; △, control perfusion with cells.

Effects of adrenergic and cholinergic effectors on efflux of ^{45}Ca from parotid cells during perfusion with Ca^{2+} -free medium

Fig. 5 shows effects of (a) 2mM -dibutyryl cyclic AMP introduced after 28 min of perfusion with Ca^{2+} -free medium, (b) $10\mu\text{M}$ -adrenaline introduced after 34 min of perfusion and (c) $10\mu\text{M}$ -isoproterenol introduced after 28 min. Each agent induced a prompt increase in the rate of efflux of ^{45}Ca , which lasted for approx. 5 min; ^{45}Ca efflux could still be induced with ionophore A23187 after cessation of the adrenaline effect (results not shown). The effect of adrenaline on ^{45}Ca efflux was blocked by $1\mu\text{M}$ -propanolol, but not by $1\mu\text{M}$ -phenolamine (results not shown), confirming that adrenergic stimulation of ^{45}Ca release is a β -adrenergic effect (see below). The enhanced efflux (in ng-atoms of Ca^{2+}/mg dry wt. of cells) amounted to 0.21 in (a), 0.19 in (b) and 0.15 in (c) (on the basis of specific radioactivity of ^{45}Ca during pre-perfusion). Amylase release was also measured with adrenaline (b) and isoproterenol (c) (results not shown). Each agent induced release of amylase, which was preceded by release of ^{45}Ca and which continued for at least 10–15 min after the rate of release of ^{45}Ca had returned to the control value.

Fig. 6 shows effects on ^{45}Ca efflux of (a) $1\mu\text{M}$ -carbamoylcholine introduced for 11 min after 32 min of perfusion with Ca^{2+} -free medium and (b) $1\mu\text{M}$ -phenylephrine introduced for 15 min after 29 min of perfusion. Each agent induced inhibition of ^{45}Ca efflux, which was maintained for the whole period of exposure. The effect of phenylephrine may have been more prompt than that of carbamoylcholine. The maximum degree of inhibition corresponds to a retention of $0.03\text{ ng-atom of Ca}^{2+}/\text{min per mg dry wt. of cells}$ with carbamoylcholine and 0.043 ng-atom with phenylephrine. The time-course of ^{45}Ca efflux with carbamoylcholine and phenylephrine contrasts sharply with the smooth curves obtained in control perfusions (Fig. 6). α -Adrenergic and cholinergic stimulation thus inhibit ^{45}Ca efflux from parotid cells.

Effects of adrenergic and cholinergic effectors and 2,4-dinitrophenol on loss of ^{45}Ca from the mitochondrial fraction of parotid pieces incubated in Ca^{2+} -free medium

Table 5 shows effects of adrenaline ($1\mu\text{M}$), isoproterenol ($1\mu\text{M}$), phenylephrine ($1\mu\text{M}$) and carbamoylcholine ($1\mu\text{M}$) on the ^{45}Ca concentration in the mitochondrial fraction isolated from parotid pieces after incubation in Ca^{2+} -free medium. In these experiments parotid pieces were preincubated for 1 h with ^{45}Ca ($2\mu\text{Ci}/\mu\text{g-atom of Ca}^{2+}$ per ml). On the basis of the data given in Fig. 3, the incorporation of ^{45}Ca into the mitochondrial fraction in this time is approx. $0.24\text{ ng-atom of medium Ca}^{2+}/\text{mg dry wt. of tissue}$. The pieces were then subjected to two successive 5 min incubations in Ca^{2+} -free medium and then incubated for 10 min in Ca^{2+} -free medium. Adrenaline, phenylephrine and carbamoylcholine were introduced for the final 10 min of incubation after which the mitochondrial fraction was isolated and assayed for ^{45}Ca . Changes in mitochondrial ^{45}Ca concentration induced by these hormones were calculated as a percentage of the control without hormone. As shown in the fourth column of Table 5, significant loss of ^{45}Ca from the mitochondrial fraction was induced by adrenaline (47%) and isoproterenol (38%); there was no significant loss with phenylephrine or carbamoylcholine. Loss of ^{45}Ca from the mitochondrial fraction of parotid pieces (39%) was induced by 2,4-dinitrophenol (0.5 mM).

The ^{45}Ca concentration in the mitochondrial fraction after incubation for 1 h with the radioactive isotope was $0.24\text{ ng-atom of medium Ca}^{2+}/\text{mg dry wt. of cells}$. This fell on incubation for 20 min in Ca^{2+} -free medium ($2\times 5\text{ min plus }1\times 10\text{ min}$, see above) to $0.12\text{ ng-atom of medium Ca}^{2+}/\text{mg dry wt. of cells}$. The exact magnitude of the additional loss induced by adrenaline, isoproterenol or 2,4-dinitrophenol cannot be calculated, because it was difficult to interrupt the

course of the experiment to measure the ^{45}Ca concentration at the beginning of the final 10 min of incubation in Ca^{2+} -free medium. The limits of this concentration were 0.12 and $0.24\text{ ng-atom of medium Ca}^{2+}/\text{mg dry wt. of cells}$ (these were the concentrations at the end of incubation in Ca^{2+} -free medium and before incubation in Ca^{2+} -free medium respectively). The limits of the losses induced by adrenaline, isoproterenol and 2,4-dinitrophenol were approx. $0.05\text{--}0.1\text{ ng-atom of medium Ca}^{2+}/10\text{ min per mg dry wt. of cells}$.

General Discussion and Conclusions

Ca^{2+} and amylase release

Table 6 summarizes the principal findings on amylase release, ^{45}Ca incorporations (cells, pieces and mitochondrial fraction) and ^{45}Ca effluxes in Ca^{2+} -free medium (cells and mitochondrial fraction). The results are compatible with a role for Ca^{2+} as a mediator of amylase release in response to α - and β -adrenergic and cholinergic effectors. The bivalent-cation ionophore A23187 enabled extracellular Ca^{2+} to stimulate amylase release; it increased both uptake of ^{45}Ca in complete medium and efflux of ^{45}Ca in a Ca^{2+} -free medium. These results suggest that the ionophore facilitated movement of Ca^{2+} across the plasma membrane, and that this was associated with amylase release. The effects of ionophore A23187 on amylase release with parotid cells were greater than those obtained with parotid slices by Selinger *et al.* (1974), but comparable with those obtained by Butcher (1975).

Amylase release by parotid cells or pieces was stimulated by α -adrenergic and cholinergic agonists, albeit weakly. This stimulation was dependent on extracellular Ca^{2+} . The most convincing demonstration of this dependence was achieved in parotid pieces. α -Adrenergic and cholinergic agonists also accelerated uptake of ^{45}Ca by parotid cells and parotid pieces and inhibited efflux of ^{45}Ca from parotid cells into a Ca^{2+} -free medium. Thus α -adrenergic and cholinergic effectors show an association between accelerated ^{45}Ca uptake and a stimulation of amylase release, which is dependent on extracellular Ca^{2+} .

Stimulation of amylase release by β -adrenergic effectors and by dibutyl cyclic AMP was not dependent on extracellular Ca^{2+} in cells or pieces, in agreement with the findings of Selinger & Naim (1970), Batzri & Selinger (1973), Harfield & Tenenhouse (1973) and Dormer & Ashcroft (1974). β -Adrenergic effectors increase parotid cyclic AMP concentrations markedly (see, e.g., Butcher, 1975), and dibutyl cyclic AMP is presumed to act as an analogue of cyclic AMP. These agents effect more marked stimulation of amylase release than do α -adrenergic or cholinergic effectors, and this might

Table 6. Summary of effects of α - and β -adrenergic agonists, carbamoylcholine, ionophore A23187, dibutyryl cyclic AMP and 2,4-dinitrophenol on amylase release and Ca^{2+} metabolism in rat parotid

Compound	Amylase release	^{45}Ca incorporation into:		^{45}Ca efflux from:	
		Cells or pieces	Mitochondrial fraction (pieces)	Cells	Mitochondrial fraction (pieces)
Ionophore A23187	Increased (dependent on external Ca^{2+})	Increased	—	Increased	—
Phenylephrine } Carbamoylcholine } Isoproterenol	Increased (dependent on external Ca^{2+})	Increased	No significant change	Decreased	No significant change
	Increased (not dependent on external Ca^{2+})	No significant change	Decreased	Increased	Increased
Adrenaline	Increased (not dependent on external Ca^{2+})	Increased (blocked by phentolamine, not by propanolol)	Increased	Increased (blocked by propanolol, not by phentolamine)	Increased
Dibutyryl cyclic AMP	Increased	—	Decreased	Increased	Increased
2,4-Dinitrophenol	Inhibited adrenaline response	Increased	Decreased	—	Increased

suggest that Ca^{2+} is not implicated in their effect. However, they inhibited uptake of ^{45}Ca into the mitochondrial fraction of parotid pieces and enhanced efflux of ^{45}Ca from the mitochondrial fraction of parotid pieces and from parotid cells into a Ca^{2+} -free medium. Therefore lack of dependence on extracellular Ca^{2+} does not exclude participation of Ca^{2+} derived from intracellular pools in their effects on amylase release.

Dormer & Ashcroft (1974) showed that adrenaline (mixed α - and β -agonist) increases ^{45}Ca incorporation into the mitochondrial fraction of parotid pieces and enhances loss of ^{45}Ca from this fraction when parotid pieces are incubated in Ca^{2+} -free medium. These findings have been confirmed, and it has been shown additionally that loss of mitochondrial ^{45}Ca is a β -adrenergic effect, whereas stimulation of cell uptake of ^{45}Ca is an α -adrenergic effect. It seems probable that adrenaline stimulates uptake of ^{45}Ca into the mitochondrial fraction through an α -adrenergic effect, although this was not established unequivocally in the present study.

Exchangeable and total Ca^{2+} pools in parotid cells and pieces

In parotid cells, approx. 15% of the total Ca^{2+} (2.9 ng-atoms/mg dry wt. of cells) exchanged with extracellular ^{45}Ca in 1–2 min. Thereafter incorporation was slow (approx. 2.5% of total Ca^{2+} /h) and incomplete after 3 h. Adrenaline increased the rate of this slow incorporation fivefold in the first 60 min, and incorporation was complete after 120 min. The additional incorporation amounted to approx. 10% of

the total cell Ca^{2+} . Whether this additional Ca^{2+} represents net uptake of Ca^{2+} or equilibration of a second more slowly exchanging pool could not be ascertained because of its modest dimension, and because it is known that adrenaline can cause release of Ca^{2+} together with amylase (Wallach & Schramm, 1971). It is clear, however, that at least 75% of parotid-cell Ca^{2+} exchanges very slowly or not at all with extracellular Ca^{2+} , and, on the basis of data of Wallach & Schramm (1971), may be located in secretory granules.

The location of the rapid and slowly exchanging pools of Ca^{2+} in parotid cells cannot be assigned with certainty. Free cytoplasmic Ca^{2+} could only account for a minute fraction (<20 pg-atoms/mg dry wt. of cells), assuming that the concentration of diffusible Ca^{2+} is no more than $10\text{ }\mu\text{M}$, as in squid axoplasm (Baker, 1972), and that the cytoplasmic volume is less than $2\text{ }\mu\text{l}$ /mg dry wt. of cells (Table 1). Important potential locations are mitochondrial and microsomal fractions, which rapidly accumulate and concentrate Ca^{2+} (Selinger *et al.*, 1970; Dormer & Ashcroft, 1974; Kanagasuntheram, 1976). The rapid release and separation of these fractions from parotid cells have not been achieved, and we have resorted in this study to the isolation of mitochondria from parotid pieces. This is less satisfactory than similar experiments with cells would have been, because equilibration of extracellular markers was slow (10–30 min), equilibration of a readily exchangeable Ca^{2+} pool (approx. 4.4 ng-atoms of Ca^{2+} out of a total pool of 17 ng-atoms/mg dry wt. of tissue) was slow (30 min), and other cell types are present. However, this study did show that rapid exchange with mitochondrial Ca^{2+} does occur (0.11 ng-atom of

Ca^{2+} /mg dry wt. of tissue in 2 min), though this incorporation is only a small fraction of the readily exchangeable Ca^{2+} (4.4 ng-atoms/mg dry wt.) or of total mitochondrial Ca^{2+} (4.8 ng-atoms/mg dry wt.). It does, however, show that there is flux of Ca^{2+} through the cytoplasmic compartment of at least 55 pg-atoms/min per mg dry wt. The bulk of the readily exchangeable Ca^{2+} in parotid pieces is thus extramitochondrial and whether or not it is intracellular or extracellular is not known.

In parotid cells, α -adrenergic effectors and carbamoylcholine induced uptake of ^{45}Ca . In Ca^{2+} -free medium, they inhibited efflux of ^{45}Ca from parotid cells. Inhibition was rapid with phenylephrine (reaching a maximum in approx. 4 min) and slower in onset with carbamoylcholine (reaching a maximum in approx. 12 min). If inhibition of Ca^{2+} efflux occurs in the presence of extracellular Ca^{2+} and is associated with a corresponding net uptake of ^{45}Ca , then the maximum uptake of ^{45}Ca can be calculated to be 0.03 ng-atom of medium Ca^{2+} /min per mg dry wt. of cells with $1\ \mu\text{M}$ -carbamoylcholine and 0.045 ng-atom of Ca^{2+} /min per mg dry wt. of cells with $1\ \mu\text{M}$ -phenylephrine. The actual rate of ^{45}Ca incorporation measured in cells with $1\ \mu\text{M}$ -carbamoylcholine was 0.018 ng-atom of medium Ca^{2+} /min per mg dry wt. of cells over 60 min. Phenylephrine was only studied with pieces and at $10\ \mu\text{M}$ where ^{45}Ca incorporation was 0.045 ng-atom of medium Ca^{2+} /min per mg dry wt. of tissue, measured over 60 min. In quantitative terms inhibition of Ca^{2+} efflux by carbamoylcholine and phenylephrine could account for the stimulation of ^{45}Ca incorporation which they induced. This interpretation assumes that the specific radioactivity of ^{45}Ca during efflux is the same as that of the medium from which it was derived during loading, and that inhibition of efflux and stimulation of incorporation have a similar time-course. These assumptions could not be verified with the present technique. If this interpretation is correct, then phenylephrine and carbamoylcholine may facilitate uptake of ^{45}Ca in parotid cells through inhibition of a Ca^{2+} pump in the plasma membrane or alternatively through inhibition of Ca^{2+} efflux from the plasma membrane itself. The participation of active mechanisms in the exclusion of Ca^{2+} from parotid cells is suggested by the marked incorporation of ^{45}Ca induced by 2,4-dinitrophenol.

The additional ^{45}Ca incorporated into parotid cells or pieces with adrenaline, phenylephrine or carbamoylcholine was apparently largely into extramitochondrial fractions. Thus the increase in the rate of incorporation (approx. 0.1–0.25 ng-atom of Ca^{2+} /10 min per mg dry wt. of parotid pieces) was sufficiently rapid to at least double the mitochondrial ^{45}Ca if incorporated into this fraction. No increase in mitochondrial ^{45}Ca was detected with any agent after 10 min of incubation. Adrenaline increased mitochondrial ^{45}Ca incorporation after 60 min (by 0.12 ng-

atom of medium Ca^{2+} /mg dry wt. of tissue), but this was only a small fraction of the total tissue incorporation (2.85 ng-atoms of medium Ca^{2+} /mg dry wt.).

In parotid cells, β -adrenergic effectors and dibutyryl cyclic AMP induced efflux of ^{45}Ca in Ca^{2+} -free medium. Wallach & Schramm (1971) found that amylase release is accompanied by release of Ca^{2+} apparently from within the secretory granule. It seems unlikely that the ^{45}Ca released in the present experiments was derived from secretory granules. The release of ^{45}Ca preceded the release of amylase and, unlike release of amylase, was short-lived, and, moreover, the Ca^{2+} of secretory granules shows negligible exchange with external ^{45}Ca (Wallach & Schramm, 1971). In parotid pieces, β -adrenergic effectors and dibutyryl cyclic AMP inhibited incorporation of ^{45}Ca into the mitochondrial fraction and induced release of ^{45}Ca from the mitochondrial fraction during incubation in Ca^{2+} -free medium. The extent of ^{45}Ca release from parotid cells with these agents was approx. 0.15–0.21 ng-atom of Ca^{2+} /mg dry wt. of cells. The duration of efflux was approx. 5 min. The extent of efflux from the mitochondrial fraction of parotid pieces during 10 min of incubation with these agents in Ca^{2+} -free medium was approx. 0.05–0.1 ng-atom of Ca^{2+} /mg dry wt. of tissue. Thus efflux of Ca^{2+} from mitochondria could account for much, if not all, of the efflux of ^{45}Ca from cells.

The incorporation of ^{45}Ca into the mitochondrial fraction of parotid pieces was decreased by 2,4-dinitrophenol in spite of a substantial increase of ^{45}Ca incorporation into the whole tissue. The efflux of ^{45}Ca from the mitochondrial fraction of parotid pieces in a Ca^{2+} -free medium was enhanced by 2,4-dinitrophenol. These effects on mitochondrial exchangeable Ca^{2+} were expected because uncouplers are known to inhibit respiration-dependent mitochondrial uptake of Ca^{2+} . In the present study the uncoupling effect of 2,4-dinitrophenol (0.5 mM) was shown by the lowered ATP concentration of parotid cells (from 7.3 ± 0.1 to 4.0 ± 0.2 nmol of ATP/mg dry wt. of cells; mean $\pm$ s.e.m. after 20 min of incubation). These effects of 2,4-dinitrophenol on parotid cells and pieces are qualitatively similar to those observed in epididymal adipose tissue, where the uncoupler decreased ^{45}Ca incorporation into the mitochondrial fraction and increased incorporation into the extramitochondrial fraction (Severson *et al.*, 1976). In adipose tissue, adrenaline lowered mitochondrial incorporation of ^{45}Ca and it was suggested that this effect might be due to an uncoupling of oxidative phosphorylation. In the present study adrenaline increased mitochondrial incorporation of ^{45}Ca , even though it produced a modest but significant fall in ATP concentration (measured in cells, from 5.2 ± 0.1 to 4.6 ± 0.1 nmol of ATP/mg dry wt., after 20 min with

1 μM -adrenaline); similar modest falls in ATP concentration were also seen in pieces with isoproterenol (1 μM), phenylephrine (1 μM) and carbamoylcholine (1 μM), i.e. with α - and β -adrenergic and cholinergic effectors. This might suggest that mechanisms other than a fall in ATP are involved in depletion of mitochondrial ^{45}Ca with β -adrenergic stimulation. One possibility is a direct effect of cyclic AMP, as suggested by Borle (1974).

General conclusions

There is evidence, in a number of secretory cells, for a role for Ca^{2+} as a mediator in stimulus-secretion coupling (Douglas & Rubin, 1961; see reviews by Matthews, 1970; Milner & Hales, 1970; Malaisse, 1973; Douglas, 1974). Some of this evidence is based on a requirement for extracellular Ca^{2+} in secretory responses. Recognition that there are secretory responses that do not depend on extracellular Ca^{2+} has focused attention on the possible participation of intracellular stores of Ca^{2+} , for example in mitochondrial and microsomal fractions (Feinstein & Schramm, 1970; Selinger *et al.*, 1970; Malaisse, 1973; Dormer & Ashcroft, 1974). The concept of Ca^{2+} as a mediator in stimulus-secretion coupling was originally envisaged as being analogous to the role of Ca^{2+} in excitation-contraction coupling in muscle (Douglas, 1974). In invertebrate muscles, contraction may be triggered by a rise in cytoplasmic free Ca^{2+} of the order of 1 μM (Portzehl *et al.*, 1964; Ashley & Ridgway, 1970). In muscle, the cytoplasmic free Ca^{2+} may be regulated by the plasma membrane, by sarcoplasmic tubules and possibly by mitochondria. Comparable systems for the regulation of cytoplasmic Ca^{2+} concentration exist in parotid (Selinger *et al.*, 1970; Dormer & Ashcroft, 1974; present paper). There is, however, little knowledge of the molecular components involved in stimulus-secretion coupling or of their location. If the system is analogous to muscle, then it is helpful to note that a rise in cytoplasmic free Ca^{2+} of, for example, 10 μM could involve addition of as little as 20 pg-atoms of Ca^{2+} /mg dry wt. of parotid cells, to the cytoplasmic compartment. This addition is miniscule in relation to total cell Ca^{2+} , total mitochondrial Ca^{2+} or total cell exchangeable Ca^{2+} , modest in relation to the rapidly exchanging pool of mitochondrial Ca^{2+} , and comparable in size with the uptake of ^{45}Ca /min induced by phenylephrine and carbamoylcholine, and to the efflux of ^{45}Ca /min from mitochondria and cells in a Ca^{2+} -free medium with isoproterenol or adrenaline. The present findings are compatible with a role for Ca^{2+} as a mediator of secretory responses in rat parotid, but more stringent evidence is required, including knowledge of the molecular mechanisms involved, their location and concentrations of Ca^{2+} in the relevant cell compartment.

The present study has shown that effects of α -adrenergic and cholinergic agonists on Ca^{2+} metabolism in rat parotid cells are similar and quite different from those of β -adrenergic effectors. Interpretation requires further study of mechanisms, and parotid is not ideal for this purpose, because of limited availability of material. It would be useful to know whether these effects are demonstrable in other cell types, more readily available in quantity. Kondo & Schulz (1976) have reported that carbamoylcholine increased the ^{45}Ca -exchangeable pool size in rat pancreatic acinar cells, whereas isoproterenol and dibutyl cyclic AMP were without effect. In unpublished work in this laboratory (Oxford) S. Foden has found that ^{45}Ca uptake by rat hepatocytes is stimulated by phenylephrine, adrenaline plus propranolol and carbamoylcholine, but not by isoproterenol or adrenaline plus phentolamine.

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References

- Amsterdam, A. & Jamieson, J. D. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2028-2032
- Amsterdam, A., Ohad, I. & Schramm, M. (1969) *J. Cell Biol.* **4**, 753-773
- Ashley, C. C. & Ridgway, E. B. (1970) *J. Physiol. (London)* **209**, 105-130
- Babad, H., Ben-Zvi, R., Bdolah, A. & Schramm, M. (1967) *Eur. J. Biochem.* **1**, 96-101
- Baker, P. F. (1972) *Prog. Biophys. Mol. Biol.* **24**, 177-224
- Batzri, S. & Selinger, Z. (1973) *J. Biol. Chem.* **248**, 356-360
- Batzri, S., Selinger, Z., Schramm, M. & Robinovitch, M. R. (1973) *J. Biol. Chem.* **248**, 361-368
- Bdolah, A., Ben-Zvi, R. & Schramm, M. (1964) *Arch. Biochem. Biophys.* **104**, 58-66
- Bergmeyer, H. U. & Brent, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 2, pp. 575-579; Academic Press, New York and London
- Bernfeld, P. (1955) *Methods Enzymol.* **1**, 149-158
- Borle, A. B. (1974) *J. Membr. Biol.* **10**, 45-66
- Burton, K. (1956) *Biochem. J.* **62**, 315-323
- Butcher, F. R. (1975) *Metab. Clin. Exp.* **24**, 409-418
- Butcher, F. R., Goldman, J. A. & Nemerovski, M. (1975) *Biochim. Biophys. Acta* **392**, 82-94
- Chance, B. & Williams, J. R. (1955) *J. Biol. Chem.* **217**, 409-417
- Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115-127
- Dormer, R. L. & Ashcroft, S. J. H. (1974) *Biochem. J.* **144**, 543-550
- Douglas, W. W. (1974) in *Secretory Mechanisms of Exocrine Glands* (Thorn, N. A. & Petersen, O. H., eds.), pp. 116-129, Munksgaard, Copenhagen

- Douglas, W. W. & Rubin, P. F. (1961) *J. Physiol. (London)* **159**, 40–57
- Feinstein, H. & Schramm, M. (1970) *Eur. J. Biochem.* **13**, 158–163
- Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Harfield, B. & Tenenhouse, A. (1973) *Can. J. Physiol. Pharmacol.* **51**, 997–1001
- Kanagasuntheram, P. (1976) Ph.D. Thesis, University of Bristol
- Kondo, S. & Schulz, I. (1976) *Biochim. Biophys. Acta* **419**, 76–92
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Malaisse, W. J. (1973) *Diabetologia* **9**, 167–173
- Malamud, D. (1972) *Biochim. Biophys. Acta* **279**, 373–376
- Mangos, J. A., McSherry, N. R., Butcher, F., Irwin, K. & Barber, T. (1975a) *Am. J. Physiol.* **229**, 553–559
- Mangos, J. A., McSherry, N. R., Barber, T., Arranitakis, S. N. & Wagner, V. (1975b) *Am. J. Physiol.* **229**, 560–565
- Mangos, J. A., McSherry, N. R. & Barber, T. (1975c) *Am. J. Physiol.* **229**, 566–569
- Matthews, E. K. (1970) in *Calcium and Cellular Function* (Cuthbert, A. W., ed.), pp. 163–182, Macmillan, London
- Milner, R. G. & Hales, C. N. (1970) in *Early Diabetes* (Camerini-Davalers & Cole, H. S., eds.), pp. 59–65, Academic Press, New York and London
- Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Randle, P. J., Denton, R. M., Pask, H. T. & Severson, D. L. (1974) *Biochem. Soc. Symp.* **39**, 75–87
- Rasmussen, H. & Tenenhouse, A. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 1364–1370
- Reed, K. C. (1973) *Biochem. Biophys. Res. Commun.* **50**, 1136–1142
- Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977
- Schramm, M. (1968) *Biochim. Biophys. Acta* **165**, 546–549
- Schramm, M. & Selinger, Z. (1975) *J. Cyclic Nucleotide Res.* **1**, 181–192
- Selinger, Z. & Naim, E. (1970) *Biochim. Biophys. Acta* **203**, 335–337
- Selinger, Z., Naim, E. & Lasser, M. (1970) *Biochim. Biophys. Acta* **203**, 326–334
- Selinger, Z., Batzri, S., Eimerl, S. & Schramm, M. (1973) *J. Biol. Chem.* **248**, 369–372
- Selinger, Z., Eimerl, S., Savion, N. & Schramm, M. (1974) in *Secretory Mechanisms of Exocrine Glands* (Thorn, N. A. & Petersen, O. H., eds.), pp. 68–79, Munksgaard, Copenhagen
- Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237
- Severson, D. L., Denton, R. M., Bridges, B. L. & Randle, P. J. (1976) *Biochem. J.* **154**, 209–223
- Simon, E. J. & Shemin, D. (1953) *J. Am. Chem. Soc.* **75**, 2520–2522
- Srere, P. A., Brazil, H. & Gonen, L. (1963) *Acta Chem. Scand.* **17**, S129–S134
- Stanley, P. E. & Williams, S. G. (1969) *Anal. Biochem.* **29**, 381–392
- Tenant, J. R. (1964) *Transplantation* **2**, 685–696
- Wallach, D. & Schramm, M. (1971) *Eur. J. Biochem.* **21**, 433–437